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# *Cecinothofagus* Nieves-Aldrey & Liljeblad (Hymenoptera, Cynipidae) is likely an endoparasitoid of the gall-maker genus *Aditrochus* Rübsaamen (Hymenoptera, Pteromalidae)

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## Abstract

*Paraulax* Kieffer and *Cecinothofagus* Nieves-Aldrey & Liljeblad (Cynipidae: Paraulacini) were long supposed to be gall-makers on southern beeches (*Nothofagus*, Nothofagaceae). Dissections of galls on *Nothofagus* Blume, suggested that *Cecinothofagus* could be instead either endoparasitoid or inquiline of *Aditrochus* larva (Chalcidoidea). We sequenced the universal *COI* barcode and Ultra-Conserved Elements (UCEs) from young larvae of *Aditrochus* collected from galls on *Nothofagus* and highlighted that one of them also contained DNA from *Cecinothofagus ibarrai* Nieves-Aldrey & Liljeblad. So far, when galls attributed to *Aditrochus* were dissected in early development stages they all contained only a single larva and no remains of other larvae. Conversely, when *Cecinothofagus ibarrai* was reared from galls on *Nothofagus*, remains of the host larva were observed inside the larval chamber. Altogether, biological observations and molecular results suggest that *Cecinothofagus ibarrai* is likely an endoparasitoid of *Adritrochus*. This result confirms the tribe Paraulacini as being entomophagous and supports the hypothesis of an ancestral parasitoid lifestyle for Cynipoidea.

## Keywords

Biology, Cynipoidea, Chalcidoidea, *Nothofagus*

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## Introduction

Paraulacini is a tribe of Cynipidae that contains two closely related genera, *Paraulax* Kieffer and *Cecinothofagus* Nieves-Aldrey & Liljeblad (Nieves-Aldrey et al. 2009). Unlike most Cynipidae that are found in the Northern Hemisphere, the six species described in Paraulacini occur in southern South-America (Argentina and Chile; Nieves-Aldrey et al. 2009). Our lack of biological knowledge on Paraulacini would have been anecdotal if the tribe was not recovered sister to all other Cynipoidea in a recent phylogenomic hypothesis proposed by Blaimer et al. (2020). Acquiring knowledge on their biology has thus become crucial to accurately infer the ancestral lifestyle of Cynipoidea.

*Paraulax* and *Cecinothofagus* were long supposed to be gall-makers on southern beeches (*Nothofagus*, Nothofagaceae) (Dalla Torre and Kieffer 1910; De Santis et al. 1993; Ronquist 1999; Csóka et al. 2005), probably by analogy with the Cynipidae of the northern hemisphere that induce gall on many plant lineages (Ronquist 1999). As of today, the biology of *Paraulax* remains unknown. Dissections of galls on *Nothofagus* suggested that *Cecinothofagus* could be instead either endoparasitoid or inquiline of larva of *Aditrochus* Rübsaamen (Chalcidoidea) (Nieves-Aldrey et al. 2009). Along with the genera *Espinosa* Gahan and *Plastobelyta* Kieffer, *Aditrochus* belongs to the tribe Melanosomellini (Pteromalidae, Ormocerinae) that only occurs in southern South America (Bouček 1988; De Santis et al. 1993). As for Paraulacini, the biology of *Aditrochus* is poorly known. *Aditrochus* is indeed supposed to be a gall-maker (Bouček 1988), while *Espinosa* and *Plastobelyta* have been considered inquilines or parasitoids of gall-makers (Bouček 1988).

In the course of a project to infer the tree of life of Chalcidoidea, we sequenced the universal *COI* barcode and Ultra-Conserved Elements (UCEs) from larvae of *Aditrochus* and highlighted that one of them contained DNA from another species that was identified as *Cecinothofagus ibarrai*. We discuss the implication of such result in the light of biological data to infer the most likely trophic relationships between *Aditrochus* and *Cecinothofagus* (Fig. 1).

## Methods

### Sampling, morphological identification and DNA extraction

Two morphologically identical larvae of a rare gall inducer *Aditrochus coihuensis* Ovruski, 1993 were extracted from two galls sampled on *Nothofagus dombeyi* (Mirb.) Ørst by JLNA [Ensenada to PN Vicente Perez Rosales, 24.xi.2013, Nieves J.L. leg.]. These larvae were databased in the collection of CBGP (Centre de Biologie pour la Gestion des Populations) and in our storage of DNA under the numbers JRAS07470_0103 and JRAS07470_0104. Larvae were independently identified as belonging to Chalcidoidea by JYR on the basis of head morphology, structure of the labrum and head chaetotaxy. DNA was extracted from the two larvae using the Qiagen DNeasy Blood and Tissue kit. A slightly modified manufacturer's protocol was used to increase DNA yield (Cruaud et al. 2019). Extractions were conducted without destruction of the larvae.

**Figure 1.** Adults of *Aditrochus coihuensis* (above) and *Cecinothofagus ibarrai* wasps (under) showing their different size. Photograph J.L. Nieves-Aldrey.

## DNA barcoding

The DNA extracted from each larva was amplified with a 2 step PCR approach targeting *COI* (universal barcode fragment) following the protocol detailed in Cruaud et al. (2017). Two overlapping fragments [FC and BR (Shokralla et al. 2015)] were amplified and sequenced on a Illumina MiSeq System (2*250 bp) together with other insects (mostly Coleoptera). Importantly, no Cynipoidea were included in the experiment. Raw data were analysed following Cruaud et al. (2017). Briefly, adapter trimming and selection of high-quality paired reads was performed with Trimmomatic (Bolger et al. 2014); paired reads were merged with FLASH (Magoc and Salzberg 2011); clustering of sequences was performed with SWARM (d=1) (Mahé et al. 2015) after dereplication with VSEARCH (Rognes et al. 2016). Only consensus sequences obtained from clusters with more than 5 identical sequences were retained for downstream analyses.

Non-coding sequences as well as sequences of endosymbionts and bioaresols were removed. FC and BR fragments that passed through all quality controls were assembled in Geneious 11.1.4 (https://www.geneious.com) to get full-length *COI* barcodes (658 bp).

### Hybrid capture of UCEs

The two DNA extracts were then used to capture about 1,400 UCEs with the 2,749 RNA probes designed by Faircloth et al. (2015) and using the protocol detailed in Cruaud et al. (2019). Extracts were included in a larger capture experiment (N samples = 96) that was sequenced on a Illumina MiSeq system, but again no Cynipoidea were included (only Chalcidoidea). Reads were analysed following Cruaud et al. (2019). Briefly, adapter trimming and selection of high-quality paired reads was performed with Trimmomatic (Bolger et al. 2014); paired reads were merged with FLASH (Magoc and Salzberg 2011) and demultiplexing was performed with a custom script (Cruaud et al. 2019). Assembly into contigs was performed with CAP3 (Huang and Madan 1999) and contigs were aligned with Lastz (Harris 2007) to the set of reference UCEs assembled from probes.

### Phylogenetic inference

Small taxa sets were assembled to assess the phylogenetic placement of the *COI* or UCE sequences obtained from the two *Aditrochus* larvae (Table 1). Alignment of *COI* sequences and individual UCEs was done with MAFFT-linsi (Katoh and Standley 2013). Alignment cleaning of individual UCEs was performed using SEQTOOLS (Mirarab et al. 2014): positions with more than 10% gaps and sequences with more than 25% gaps were removed. Three rounds of Treeshrink with b=10 (Mai and Mirarab 2018) were also performed on individual UCE trees to remove abnormally long branches. Trees were built with IQ-TREE 2.0.6 (Minh et al. 2020) from the *COI* data set and from the concatenated UCE data set (no partition) with best fit models selected by ModelFinder (BIC criterion) (Kalyaanamoorthy et al. 2017). FreeRate models with up to ten categories of rates were included in tests for the UCE data set, but only common substitution models were tested for *COI*. The candidate tree set for all tree searches was composed of 98 parsimony trees + 1 BIONJ tree and only the 20 best initial trees were retained for NNI search. Statistical support of nodes was assessed with ultrafast bootstrap (UFBoot) (Minh et al. 2013) with a minimum correlation coefficient set to 0.99 and 1,000 replicates of SH-aLRT tests (Guindon et al. 2010).

## Results

### DNA barcoding

Only one *COI* sequence was obtained from the first larva (JRAS07470_0103; BR only; 88 sequences in the SWARM cluster). For the second larva (JRAS07470_0104), the exact same sequence was obtained (BR only; 6 sequences in the SWARM cluster) but,

in addition, another sequence that had a positive match on NCBI with *Cecinothofagus ibarrai* Nieves-Aldrey & Liljeblad, 2009 (100% identity; query cover 91%) was also found (FC+BR with, respectively, 127 and 240 sequences in the SWARM clusters). This second sequence corresponds exactly to the sequences of *Cecinothofagus ibarrai* deposited in Genbank by the describers, which cross validated both sequences. Sequences obtained from the two larvae were analysed with Genbank sequences (Table 1) to produce the phylogenetic tree shown in Fig. 2a (best fit model = TIM+F+G4).

## Capture of UCEs

For a large number of reference UCEs, two contigs instead of one were recovered in the second larva of *Aditrochus* (JRAS07470_0104). These contigs were blasted against a subset of 400 UCEs that were successfully captured from both *Cecinothofagus ibarrai* (by Blaimer, et al. 2020) and the first larva. 712 contigs retrieved from the second larva had a hit for 392 of these 400 UCEs. For 62 UCEs (on 392), contigs had a hit only with *Aditrochus*; for 17 UCEs, contigs had a hit only with *C. ibarrai* and for the remaining 313 UCEs two contigs were present in the second larva that either matched with *Aditrochus* or *C. ibarrai*. The phylogenetic tree obtained from a larger set of taxa (n=12; Table 1) and 310 UCEs (90% complete matrix; 91,607 bp) is shown in Fig. 2b (best fit model = GTR+F+I+G4).

**Table 1.** Taxa included in phylogenetic analyses.

| Classification | Species | Accession *COI* / UCEs | Source *COI* /UCEs | Nb UCEs (after Treeshrink) |
|---|---|---|---|---|
| CHAL: Pteromalidae: Ormocerinae | *Aditrochus coihuensis* [JRAS07470_0103 larva1] | OP539441 / SAMN31038493 | This study | 266 |
| CHAL: Pteromalidae: Ormocerinae | *Aditrochus coihuensis* [JRAS07470_0104 larva2] | OP539442 / SAMN31038494 | This study | 246 |
| CHAL: Pteromalidae: Ormocerinae | *Espinosa nothofagi* | n.a. /SAMN31038496 | n.a. /This study | 191 |
| CHAL: Pteromalidae: Epichrysomallinae | *Odontofroggatia* sp. | HM770633 /n.a. | Cruaud et al. 2011 /n.a. | n.a. |
| CHAL: Ormyridae | *Ormyrus rosae* | KM561583 /n.a. | Unpublished /n.a. | n.a. |
| CYNI: Cynipidae: Paraulacini | *Cecinothofagus ibarrai* | FJ998298 / SAMN15608859 | Nieves-Aldrey et al. 2009 / Blaimer et al. 2020 | 266 |
| CYNI: Cynipidae: Paraulacini | *Cecinothofagus ibarrai* [endoparasitoid of JRAS07470_0104] | OP539440 / SAMN31038494 | This study | 248 |
| CYNI: Cynipidae: Cynipini | *Disholcaspis lasia* | n.a. /SAMN06672405 | n.a. /Branstetter et al. 2017 | 268 |
| CYNI: Cynipidae: Cynipini | *Disholcaspis quercusmamma* | n.a. /SAMN06672406 | n.a. /Branstetter et al. 2017 | 275 |
| CYNI: Ibaliidae | *Ibalia anceps* | DQ012641 / SAMN06672424 | Unpublished /Branstetter et al. 2017 | 275 |
| CYNI: Figitidae: Eucoilinae | *Leptopilina japonica* | MK268803 / SAMN15608914 | Unpublished /Blaimer et al. 2020 | 149 |
| CYNI: Cynipidae: Pediaspidini | *Pediaspis aceris* | AY368929 / SAMN15608898 | Nylander et al. 2004 / Blaimer et al. 2020 | 96 |
| CYNI: Cynipidae: Diastrophini | *Periclistus brandtii* | KF936633 / SAMN31038495 | Malm and Nyman 2015 / This study | 264 |
| CYNI: Figitidae: Aspicerinae | *Prosaspicera sp.* | n.a. /SAMN06672413 | n.a. /Branstetter et al. 2017 | 265 |

CHAL= Chalcidoidea; CYNI=Cynipoidae.

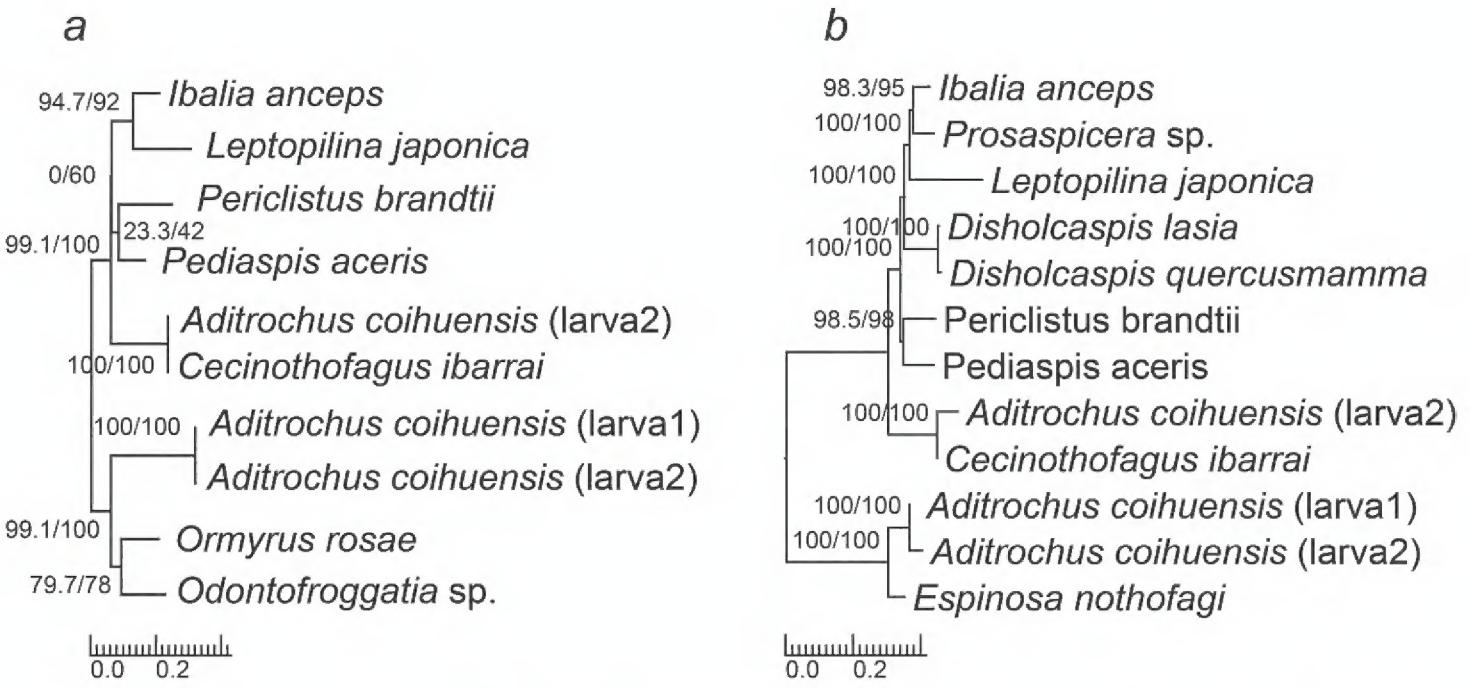


**Figure 2.** Phylogenetic trees. **a** *COI* barcode tree **b** UCE tree (obtained from the concatenation of 310 UCEs after 2 rounds of treeshrink on each individual UCEs). Statistical support (SHaLRT/UFBoot) are shown at nodes. Accession numbers for sequences used in the analyses are listed in Table 1.

## Discussion

Cynipids reared from galls on *Nothofagus* (*Paraulax* and *Cecinothophagus*) have long been supposed to be gall inducers (Dalla Torre and Kieffer 1910; De Santis et al. 1993; Ronquist 1999; Csóka et al. 2005). Gall dissection by Nieves-Aldrey et al. (2009) (see also Figs 3, 4) suggested that species of *Cecinothofagus* were instead parasitoids or lethal inquilines within galls induced by species of *Aditrochus*.

Larvae assigned to *Aditrochus* were observed by one of us (JLNA) in dozens of dissected galls collected on *Nothofagus* species in Chile in field campaigns from the years 2005, 2006, 2012, 2013 and 2014. In all cases, the galls dissected in early development stages contained only a single larva occupying the central larval chamber in the gall (Fig. 3). Furthermore, no remains of other larvae were present which confirmed that the larvae were gall inducers and not parasitoids. Here we confirm that these larvae belong to Chalcidoidea on the basis of morphology and both DNA barcoding and sequencing of UCEs. Although the biology of Melanosomellini is poorly known (only half of the 30 genera have reliable host records; Noyes 2019), most of them are considered to be gall makers (Noble 1941). *Trichilogaster acaciaelongifoliae* (Froggatt, 1892) has even been used to control the invasive *Acacia longifolia* (Andr.) Willd. in South Africa. Melanosomellini are associated with eight plant families that originated in the southern hemisphere: Myrtaceae (7 genera), Fabaceae Mimosoideae (5), Fagales [Nothofagaceae (3) and Casuarinaceae (2)], Malvales [Malvaceae (2) and Elaeocarpaceae (1)], Celastraceae (1) and Apocynaceae (1). However, *Brachyscelidiphaga* appears to be an inquiline in galls of *Apiomorpha* Rübsaamen (Hemiptera, Eriococcidae) on *Eucalyptus* L'Hér. (Bouček 1988). Therefore, our results are in agreement with the most common biology found in Melanosomellini.

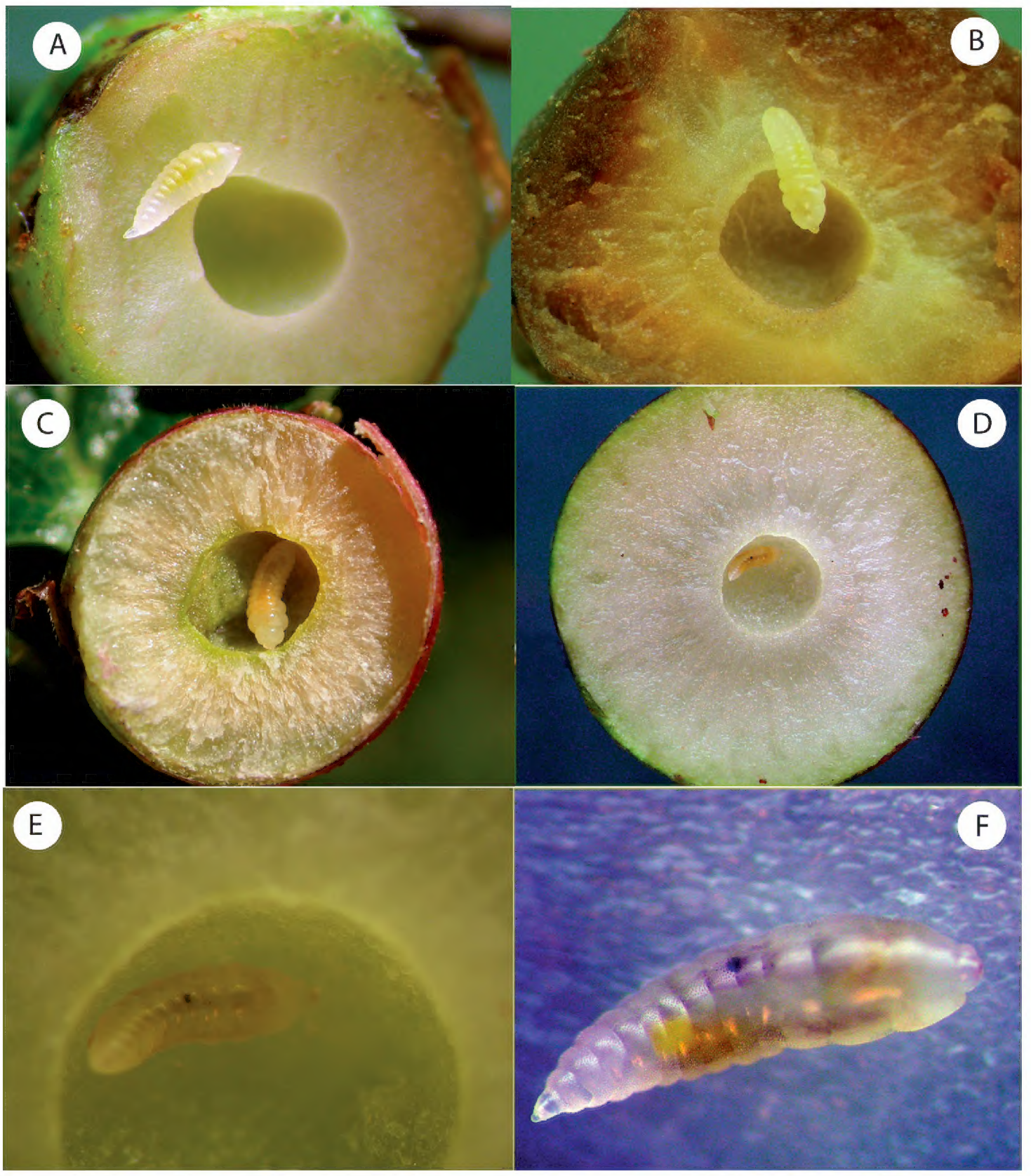


**Figure 3.** Cross section of galls of *Aditrochus* species on *Nothofagus* showing the central larval chamber and the gall inducer *Aditrochus* larva (note the absence of remains of other larvae inside the chamber). **A, B** *Aditrochus coihuensis* **C** *Aditrochus fagicolus* **D–F** *Aditrochus coihuensis* larva paralyzed by an endoparasitoid (likely *Cecinothofagus ibarrai*). Photographs J.L. Nieves-Aldrey.

Conversely, when *Cecinothofagus ibarrai* was reared from galls on *Nothofagus*, remains of the host larva were observed inside the larval chamber (Fig. 4). Here, we show that one larva of *A. coihuensis* (JRAS07470_0104) also hosted the DNA of *Cecinothofagus ibarrai.* From all these results, we can conclude that *Cecinothofagus ibarrai* was likely an endoparasitoid of this larva. This result confirms that the early

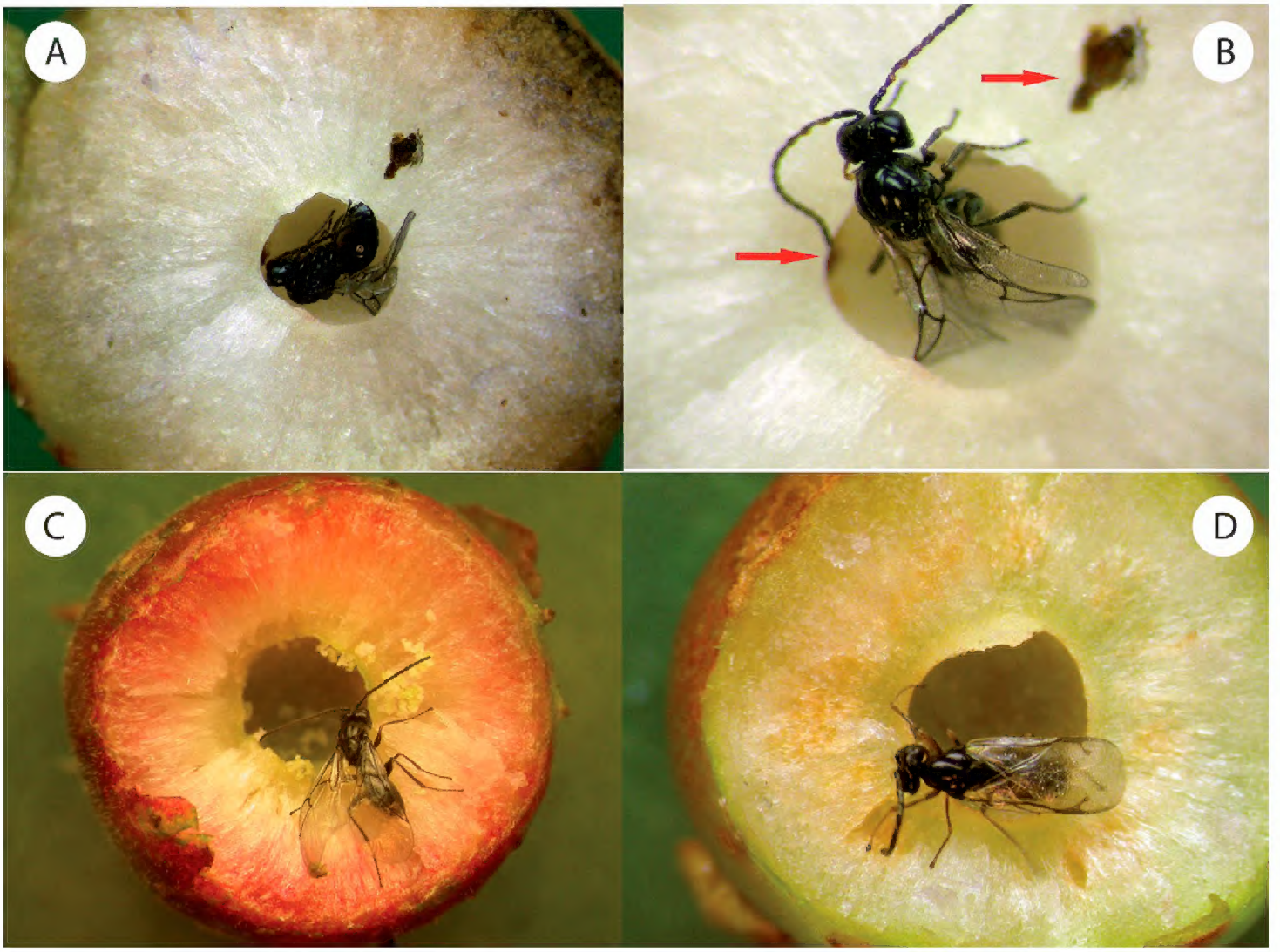


**Figure 4.** Cross sections of galls of *Aditrochus* species on *Nothofagus* showing emergences of the gall inducer *Aditrochus* adult and the endoparasitoid *Cecinothofagus* adult **A, B** *Cecinothofagus ibarrai* (Cynipidae) (note the remains of the host larva inside the larval chamber) **C** *Cecinothofagus ibarrai* emerged from a gall of *Aditrochus gnirensis* on *Nothofagus antarctica* **D** Adult *Aditrochus coihuensis* emerged from its gall on *Nothofagus dombeyi*. Red arrows show the remains of the host larva. Photographs J.L. Nieves-Aldrey.

evolution of cynipoids may be entomophagous in nature (Blaimer et al. 2020). In this study where the ancestral lifestyle of Cynipoidea was estimated to be either inquiline or parasitoid, our results may contribute to remove ambiguity.

To conclude, our study demonstrated that the usual trophic interactions observed in northern hemisphere on Fagaceae (cynipids are gall makers and pteromalids are parasitoids) is reversed in the southern hemisphere on Fagaceae (pteromalids are gall makers and cynipids are parasitoids or inquilines) … … a bit like water drains the other way Down Under!

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